Effect of prenatal stress on subsequent response to mixing stress and a lipopolysaccharide challenge in pigs

D. C. Lay, Jr., H. G. Kattesh, J. E. Cunnick, M. J. Daniels, G. Kranendonk, K. A. McMunn, M. J. Toscano and M. P. Roberts

J ANIM SCI 2011, 89:1787-1794.
doi: 10.2527/jas.2010-3612

Effect of prenatal stress on subsequent response to mixing stress and a lipopolysaccharide challenge in pigs[1]

D. C. Lay Jr.,*[2] H. G. Kattesh,† J. E. Cunnick,‡ M. J. Daniels,§ G. Kranendonk,# K. A. McMunn,* M. J. Toscano,*[3] and M. P. Roberts†

*ARS-USDA, Livestock Behavior Research Unit, West Lafayette, IN 47980; †Department of Animal Science, University of Tennessee, Knoxville 37996; ‡Department of Animal Science, Iowa State University, Ames 50014; §Department of Statistics, University of Florida, Gainesville 32611; and #Department of Foetal and Neonatal Biology, Utrecht University, 3508 Utrecht, the Netherlands

ABSTRACT: Sows subjected to prenatal stress have been found to produce offspring that have altered responses to stress. Our objective was to determine if exposing a sow to stress would alter the response of the offspring to lipopolysaccharide (LPS) at 2 mo of age or their response to mixing stress at 4 mo of age. Sow treatments consisted of intravenous injections of ACTH (1 IU/kg of BW), exposure to rough handling for a 10-min duration (rough), or no treatment (control) once per week from d 42 to 77 of gestation. At 2 mo of age, pigs from each treatment, 1 per litter (n = 21, 17, and 15 for the ACTH, rough, and control treatments, respectively), were challenged with 2 μg of LPS/kg of BW or saline, or served as a noninjected control. Their behavioral response to a human approach test and salivary cortisol were measured. At 4 mo of age, 1 pig from each treatment (n = 14, 14, and 15 for the ACTH, rough, and control treatments, respectively) was taken from its home pen and placed in a pen of unfamiliar pigs. At this time, a punch biopsy wound (6 × 6 mm) was created to measure the ability of the pig to heal the wound. At this same time, each pig received a 1-mL intramuscular injection of 20% ovine red blood cells (oRBC), and then a second injection of oRBC at 21 d postmixing. Blood samples were collected 3 times per week for 2 wk and then once a week for 4 more weeks. Blood samples were analyzed for cortisol, porcine corticosteroid-binding globulin, antibody response to oRBC, and nitric oxide production by macrophages. Behavior was recorded during the first 5 d after mixing. All pigs in the LPS challenge responded with characteristic sickness behavior; however, pigs in the rough treatment showed less sickness behavior than those in the other 2 treatments ($P < 0.05$). Maternal stress treatment did not affect ($P < 0.43$) salivary cortisol. Pigs from all treatments responded similarly to mixing stress with regard to cortisol, porcine corticosteroid-binding globulin, antibody titers, nitric oxide production, and hematology measures, and all pigs experienced the same amount of aggression in response to mixing. Without altering peripheral measures of stress responsivity, prenatal stress enhanced the ability of pigs to cope with a simulated immune challenge, which could prove to be an adaptation to challenging environments.

Key words: corticosteroid-binding globulin, cortisol, immune, prenatal, stress, swine

J. Anim. Sci. 2011. 89:1787–1794
doi:10.2527/jas.2010-3612

INTRODUCTION

Prenatal stress, the stress imposed on a pregnant dam, influences her subsequent offspring by altering characteristics of the hypothalamic-pituitary-adrenal (**HPA**) axis (Haussmann et al., 2000; Kanitz et al., 2003; Otten et al., 2010). Increases in maternal cortisol, through either exogenous ACTH (Haussmann et al., 2000) or social stress (Jarvis et al., 2006), caused offspring to have an increased cortisol response to mixing stress. In contrast, maternal ingestion of hydrocortisone acetate (**HCA**; Kranendonk et al., 2006a) did not increase the cortisol response of the offspring to mixing; in fact, these pigs were shown to have decreased basal

[1]This work was supported in part by a USDA-National Research Initiative Competitive Grant (No. 2000-02001; Washington, DC) and the USDA-Livestock Behavior Research Unit (West Lafayette, IN). Mention of a trade name, proprietary product, or specified equipment does not constitute a guarantee or warranty by the USDA and does not imply approval to the exclusion of other products that may be suitable.

[2]Corresponding author: Don.Lay@ars.usda.gov

[3]Current address: University of Bristol, School of Veterinary Science, Churchill Building, Langford, Bristol, North Somerset, BS40 5DU, UK.

Received October 18, 2010.
Accepted January 18, 2011.

cortisol. To further blur the issue, some research (Couret et al., 2009; Otten et al., 2010) has found no effect of prenatal stress on the cortisol response of the pig. Although prenatal stress research on HPA function has found diverse results, what is clear is that most studies find some alteration of the HPA axis in glucocorticoid response (mentioned above), binding globulins (Otten et al., 2010), measures of adrenal physiology (Haussmann et al., 2000), or HPA axis receptors (Haussmann et al., 2000; Kanitz et al., 2003).

Activation of the HPA axis is known to affect immune function adversely; thus, it is important to understand if prenatal stress also affects the ability of pigs to combat immune challenges. Most prenatal stress studies in swine have focused on the HPA axis of the neonatal pig, with limited emphasis on immune capability. Thus, the focus of this research was to examine the interaction between the HPA response and immune system function. Therefore, in our first test, we induced sickness behavior in the pigs by using the lipopolysaccharide (**LPS**) challenge model (de Groot et al., 2007), and in our second test, prenatally stressed pigs were subjected to stress while simultaneously challenged with healing a small wound and mounting an antibody response.

MATERIALS AND METHODS

The procedures reported herein were approved by the Purdue University Animal Care Committee. The treatments as applied to the sows have been reported previously (Lay et al., 2008); however, the pigs used in this study and the data presented are unique to this report.

Sow Treatments

Prenatal stress was created using our previously established methods (Lay et al., 2008) by subjecting gestating sows to injections of ACTH or rough handling during gestation. All sows (n = 64, parity 2 to 4) were housed in standard gestation stalls (2.21 × 0.61 m) within the same room. Sows (Landrace × Yorkshire) were either roughly handled (rough treatment, n = 21) during gestation, given an injection of ACTH (ACTH treatment, 1 IU/kg of BW, n = 21), or left undisturbed (control treatment, n = 22). Once daily during gestation, all sows were fed approximately 2.3 kg of a standard corn- and soybean meal-based gestation diet; intake was increased gradually to ad libitum after farrowing. All sows were allowed to farrow naturally. All sows were farrowed side-by-side in standard farrowing crates (1.4 × 2.5 m) with metal bars confining the sow within a 0.53 × 2.1 m area in the center of the crate. Pigs had access to the entire pen area. Expanded metal flooring covered the entire crate except for a 30.5 × 121.9 cm section of one of the side areas that had solid flooring. Each litter received heat from a 250-W lamp suspended above the solid floor area. Sows that tested not pregnant after allocation to treatment or that farrowed fewer than 6 pigs were not used in the study; thus, the final sample sizes were rough treatment, n = 20; ACTH treatment, n = 16; and control treatment, n = 20.

To invoke a state of prenatal stress, we handled the sows roughly in the rough treatment on d 42, 49, 56, 63, 70, and 77 of gestation. This rough handling consisted of herding the sows down an alley (1.3 × 15.5 m) in the gestation barn for 10 min, with each sow receiving a mild electric shock from a battery-operated livestock prod (Hot Shot, Savage, MN) 3 times at approximately 1, 3, and 7 min. This rough handling regimen was designed to simulate a moderate, but real, stressor that sows might experience during their lives. The second treatment consisted of our ACTH injection model, which was developed from previous research (Haussmann et al., 2000). The model of prenatal stress using ACTH injection was developed because, unlike subjecting a sow to a rough handling stress, injections of ACTH create a more uniform plasma cortisol response among sows without the amount of psychological stress caused by rough handling. These sows received intravenous injections of ACTH (1 IU/kg of BW, 1-39 Corticotropin A: A6303, Sigma Chemical Co., St. Louis, MO) on the same day of gestation that the roughly handled sows received their treatment. The control treatment allowed sows to remain in gestation stalls undisturbed. The entire study was conducted in 4 replications of n = 16, 18, 10, and 12, respectively, for sows entering the study. All pigs were weaned at approximately 3 wk of age and placed in mixed-sex groups of 6 (2 per dam treatment) into nursery pens. Group structure was maintained when the pigs were moved from the nursery at approximately 8 wk of age and placed into their growing-finishing pens for the remainder of the study.

LPS Challenge

At 9 wk of age, 53 pigs from separate litters, balanced by BW and sex, were subjected to an LPS challenge test. Pigs born to dams in the ACTH, rough, or control treatment (n = 21, 17, and 15, respectively) were injected intravenously with either 2 μg of LPS/kg of BW (*Escherichia coli*, serotype O111:B4, Sigma Chemical Co.) or saline, or they served as noninjected controls (n = 20, 15, and 18, respectively). Of the 18 pigs injected with LPS, 6 were from dams in the ACTH treatment, 8 were from dams in the rough treatment, and 6 were from dams in the control treatment. This test was administered to determine if prenatally stressed pigs had an altered sensitivity to an inflammatory stimulus. Injected pigs were caught from their pen and held on their backs in a V-trough while the treatment was injected intravenously in an ear vein. Noninjected control pigs were caught from their pen and held on their backs in the V-trough, but were not injected. Injection time was considered time 0. Saliva (1 mL) was collected for cortisol analysis at time −1, −0.5, 1, and 6 h relative to injection, and within 2 h of

sampling, it was placed on ice until frozen. Saliva collection was accomplished by allowing the pig to chew on a piece of cotton that was tied to a string. The pigs freely chose to chew on the cotton and did not need to be restrained. An approach test was used to measure the relative sickness of each pig at −0.5, 1, and 4 h relative to injection. The approach test was conducted by having 1 person (always the same) enter the pen of the pig and stand in the middle. The pig was considered to have approached when it touched the observer with its snout. The test was conducted for a maximum of 2 min, and the latency for the subject pigs to approach was recorded.

Mixing Stress

Up to 16 wk of age, pigs had been housed in stable groups of 6 (2 per maternal treatment of ACTH, rough, or control). At this age, pigs were subjected to mixing stress whereby 1 pig (n = 14, 14, and 15, for the ACTH, rough, and control treatments, respectively; groups included 10, 10, and 5 females, respectively) was taken from its home pen and placed into another pen from which a pig had been removed. Thus, a group size of 6 was maintained and each group of 6 had 1 subject pig added that was unfamiliar to the 5 pigs. Allocation of subject pigs to pens was balanced by BW, sex, and prenatal treatment. Pigs per treatment in the pen were maintained. On the day that test pigs were mixed, a small wound was created in the skin of the left hind quarter by using a punch biopsy (6 × 6 mm, product 33-36, Miltex Instrument Co., Lake Success NY). At this same time, each subject pig received a 1-mL intramuscular injection of 20% ovine red blood cells (**oRBC**). They received a second injection of oRBC at 21 d postmixing.

Punch biopsies were evaluated on d 0, 2, 4, 7, 9, 11, 14, 16, and 22 after mixing, following our previous published procedures (Haussmann et al., 2000). Briefly, evaluations consisted of a direct visual assessment to record inflammation and healing, and the biopsy wound was photographed from a distance of 30.5 cm from the wound for later wound scoring by 3 observers blinded to the treatments. Observers scored each biopsy wound from the photograph on a scale of 1 to 4, with 1 indicating no inflammation, 2 indicating slightly pink around the wound, 3 indicating a large red or pink ring around the wound, and 4 indicating a deep red ring around the wound with pus. In addition, a ruler was placed beside the wound for photography, and the wound length and extent of inflammation were measured (maximum width and length). Behavior was recorded for the first 5 d postmixing by time-lapse video (1 frame/0.4 s). Blood samples were collected from subject pigs on d 0, 2, 4, 7, 9, 11, 14, 21, and 35 postmixing. On d 0, all pigs were blood sampled and then placed into their respective pens beginning at 1100 h. Subsequent samples were collected to match the d 0 sampling times. To collect blood, each pig was snared in its pen and 3 separate intravenous blood samples were collected. One 3-mL blood sample was collected into an evacuated tube containing K_3EDTA (7.5%) for cortisol and porcine corticosteroid-binding globulin (**pCBG**) analysis, and immune cell population quantification. Evacuated tubes containing heparin (143 USP units) were used to collect 10 mL of blood to test the ability of macrophages to produce nitric oxide. Evacuated tubes without anticoagulant were used to collect 5 mL of blood for measurement of antibody titers to oRBC. Blood samples to be analyzed for cortisol and pCBG were immediately placed in an ice bath until they were centrifuged (650 × *g* for 15 min) at 5°C within 2 h of sampling. Blood samples used to measure nitric oxide production were kept on ice until the assay was initiated 12 h later. Blood samples to measure antibody titers to oRBC were kept chilled overnight, and serum was separated and stored at −20°C until analysis.

Data Analysis

Cortisol concentrations (nmol/L) were determined on duplicate samples using standard RIA double-antibody kits (DiaSorin Inc., Stillwater, MN) previously validated for swine plasma (Daniel et al., 1999). Samples were rerun if duplicates differed by more than 5%. Precision and accuracy of this assay were evaluated in triplicate using a swine plasma pool containing 100 ng/mL of cortisol, resulting in an intraassay CV of 7.1% and an interassay CV of 7.9%. The concentration of pCBG (mg/L) in plasma was measured by a direct ELISA, as described by Roberts et al. (2003). The intraassay CV was 9% and the interassay CV was 13.7%. The free cortisol index was calculated using the ratio of plasma total cortisol to pCBG concentration (le Roux et al., 2002), as validated for swine (Adcock et al., 2006).

Behavioral data were collected from video recordings using the Observer program (Noldus Information Technology, Leesburg VA). Three trained observers collected data from 30-min segments of video at 0900, 1300, and 1700 h for 5 d. The mixing and physiological sampling procedures were finished at approximately 1300 h; thus, the first behavioral observation was at 1700 h on d 0. Therefore, on d 0, data for only the 1700-h period was collected, and on d 5, data for only the 0900- and 1300-h periods were collected, for a total of 4 observations each at 0900, 1300, and 1700 h over the 5-d period. Agreement among the observers was verified for count data by performing 2 single-sample χ^2 tests on aggression received and aggression initiated. The probability of rejecting the null hypothesis was $P > 0.2$; thus, there were no differences in the scores for the 3 observers. The unbiased reliability of the 3 observers was $r_3 = 0.97$, computed from the appropriate ANOVA model. From the video recordings, mutually exclusive states were recorded as resting, sitting, moving, and eating. Agonistic behavior was recorded as aggression and paralleling. A modifier was included to designate whether the test pig was the initiator. Ag-

gression was defined as the behavior exhibited by the pig when it thrust its mouth or head at the shoulders, neck, or head of another pig. The force of the contact could vary from a glancing contact to a forced contact that moved the recipient. Paralleling was defined as the interaction between 2 or more pigs in which they stood and moved shoulder to shoulder, facing in either the same or the opposite direction. It was defined as having stopped when the pigs stopped moving for more than 5 s, or when they first moved apart from one another for more than 5 s.

An aliquot of whole blood (approximately 60 μL) was analyzed using an automated hematology system (QBC Vet Autoread Hematology Analyzer, IDEXX Laboratories Inc., Westbrook, ME) to quantify hematocrit, hemoglobin, mean corpuscular hemoglobin, white blood cells, granulocytes, lymphocyte-monocyte population (the system could not reliably distinguish these 2 populations, so they were included as one), and reticulocytes. To test the ability of the macrophages of the pigs to produce nitric oxide, supernatants collected from isolated peripheral blood leukocytes cultured with and without LPS (10 and 20 μg/mL of media) were tested for nitrite based on the Griess reaction (Chou et al., 1996). The absorbance at 550 nm was measured using a microplate reader.

To measure the antibody response to oRBC, serum collected from pigs was heated at 56°C for 30 min before testing its ability to inactivate the complement. Heat-inactivated serum was serially diluted 1:2 with PBS in a 96-well round-bottom plate. Each sample was run in duplicate. An equal volume of washed 2% oRBC was plated with each dilution and in negative control wells. The sealed plate was incubated at room temperature in the dark for 24 h, and agglutination was determined using the agglutination drip method (Snyder et al., 1981). The titer was recorded as the $\log_2$ of the reciprocal of the greatest dilution giving a positive result.

Statistical Analysis

Data are presented as means ± SE. Data were analyzed using Proc Mixed with either a first-order autoregressive or heterogeneous first-order autoregressive covariate structure as appropriate (SAS Inst. Inc., Cary, NC), with a longitudinal analysis for data collected over time. The models included the fixed effects of repetition, day, sex, dam treatment, and LPS treatment (only for LPS tests), with pig included as a random effect in the model. Behavioral data collected during mixing were analyzed as compositional data using a log transformation, and models were fit in Proc Mixed with a correlation structure to capture the correlation among behaviors and over time. When significant differences ($P < 0.05$) were detected, appropriate adjustments (Tukey-Kramer or Bonferroni) were used for pairwise comparisons between treatments. Data for punch biopsy wound scores and LPS approach times were analyzed using the Kruskal-Wallis test. Data were considered to exhibit a trend when $P > 0.05$ and $P < 0.10$.

RESULTS

Pig Response to LPS

The main effect of prenatal stress treatment on approach times was not different ($P = 0.36$), but the interaction between prenatal stress treatment and LPS treatment was significant ($P = 0.0001$). Injections of LPS caused a sickness response in pigs from all treatments ($P = 0.0001$), as measured by latency to approach the human observer in the pen (data not shown). Saline-injected and behavioral control pigs all responded similarly, with approach times of less than 20 s at all test times (data not shown). Before LPS injections, pigs would approach the observer within 17.25 ± 5.95 s; however, at 1 h postinjection, pigs that received LPS had an approach time of 93.85 ± 9.55 s, as opposed to saline-injected and behavioral control pigs, which approached within 10.00 ± 2.00 s. This same pattern was observed during the approach test at 4 h postinjection. Of the pigs injected with LPS, pigs in the rough treatment had a much shorter latency to approach at 1 h postinjection compared with pigs in either the ACTH or control treatment (Figure 1; $P < 0.05$). These differences were not present at 4 h postinjection because all pigs had quicker approach times.

Prenatal stress treatment did not affect salivary cortisol ($P = 0.43$). Response to the LPS treatment was significant ($P = 0.004$), with salivary cortisol tending to increase in response to LPS ($P = 0.07$; sampling time at −1 h of 1.97 ± 0.43, to 6.43 ± 1.72 nmol/L 1 h later, to 4.26 ± 1.38 nmol/L 6 h later; data not shown) as well as saline ($P = 0.04$; sampling time at −1 h of

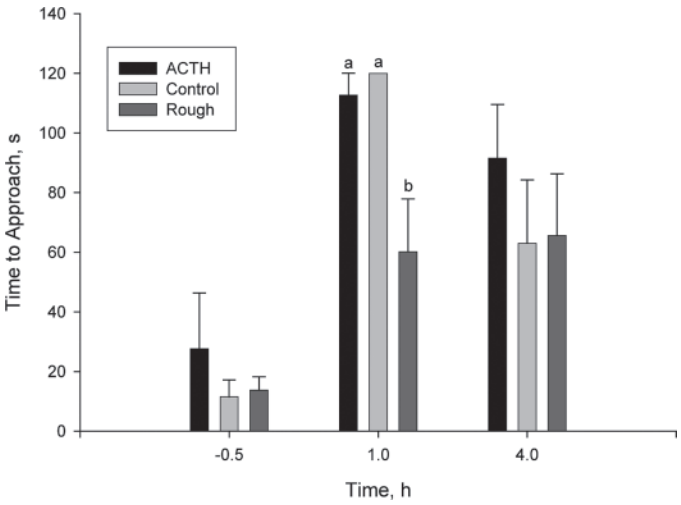

Figure 1. Mean (±SE) latency for pigs to approach the human observer in the human approach test. Pigs were injected with 2 μg of lipopolysaccharide (LPS)/kg of BW (*Escherichia coli*, serotype O111:B4) at time 0. n = 6 for the ACTH treatment, n = 6 for the control treatment, and n = 8 for the rough treatment. None of the 6 control pigs approached during the test at 1 h. [a,b]Means within time with different letters differ ($P < 0.05$).

0.86 ± 0.20, to 1.95 ± 0.45 nmol/L 1 h later, to 0.79 ± 0.44 nmol/L 6 h later). The salivary cortisol of behavioral control pigs remained constant at 2.29 ± 0.71 nmol/L between baseline and 1 h posthandling.

Pig Response to Mixing

Stress Response. Prenatal stress treatments did not alter the stress response of the pig to mixing. Pigs exhibited a heightened cortisol response to mixing on d 0, which decreased by d 35: 112.0 ± 22.9 and 77.8 ± 13.7 nmol/L, respectively ($P = 0.01$; data not shown). However, they did not show differences because of treatments ($P = 0.81$), nor were treatment differences exhibited for plasma concentrations of pCBG ($P = 0.61$), with pigs experiencing essentially static concentrations of 5.11 ± 0.07 mg/L overall except for d 0, when all treatments were relatively greater, at 5.97 ± 0.05 mg/L ($P = 0.001$). A sex difference was noted, with females having greater concentrations of pCBG than males: 5.44 ± 0.09 and 4.66 ± 0.11 mg/L, respectively. The free cortisol index also did not differ in response to treatment ($P = 0.44$; Figure 2) but did show a trend similar to plasma cortisol, with an increase on d 0 and a decline ($P = 0.007$) to the end of the study period.

Behavior. The activity of pigs was similar among all treatments during the week after mixing ($P = 0.21$; data not shown). Pigs spent similar amounts of time resting, moving, eating, and sitting. The amounts of aggression received, aggression initiated, and total aggression were the same for pigs in all treatments ($P > 0.50$). On d 1, pigs received a total of 7.6 ± 1.9 aggressive interactions during the 30-min observation period, compared with 2.4 ± 0.7 on d 5 after mixing. Pigs in all treatments performed a similar amount of paralleling behavior as well ($P = 0.54$). Of each 30-min observation period, pigs spent only 23.9 ± 3.2 s paralleling.

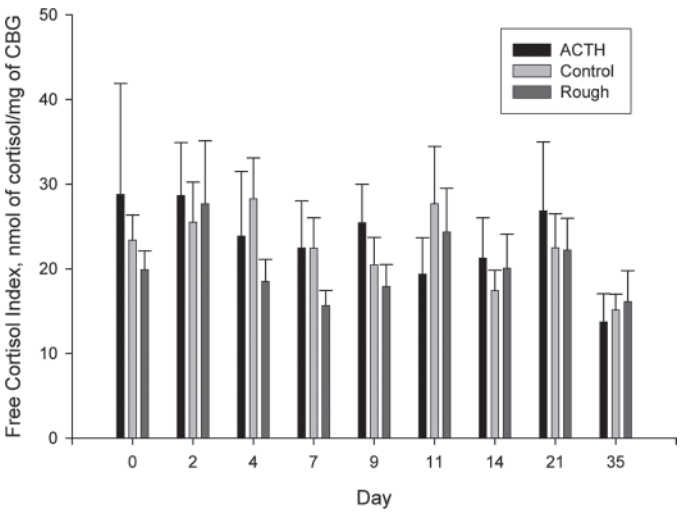

Figure 2. Means (±SE) for the free cortisol index (nmol/mg) in response to mixing of pigs that were born to sows that received an ACTH injection (ACTH treatment; 1 IU/kg of BW), served as controls (control treatment), or were roughly handled (rough treatment). The free cortisol index was calculated by dividing plasma cortisol concentrations (nmol/L) by porcine corticosteroid-binding globulin concentrations (CBG, mg/L) of pigs sampled on d 0, 2, 4, 7, 9, 11, 14, 21, and 35. n = 14 for the ACTH treatment, n = 15 for the control treatment, and n = 14 for the rough treatment.

Immune Competence. The main effect of prenatal stress treatment was not significant for punch biopsy scores ($P = 0.06$); however, there was a significant time × treatment interaction ($P = 0.02$). Punch biopsy wound scores for pigs from all treatments did not differ over the first few days after mixing ($P > 0.13$); however, by d 7, the wounds of control pigs were healed more than those of pigs in the ACTH treatment, but not pigs in the rough treatment ($P = 0.05$; Figure 3). However, this trend was reversed on d 16 ($P = 0.05$). Few overall treatment differences were found for the hematology measures assayed (Table 1; $P > 0.10$). Wound length and inflammation did not differ by treatment ($P > 0.70$). However, percentages of granulocytes and lymphocytes-monocytes did show a treatment × time interaction, which was characterized by pigs in the ACTH treatment having more lymphocytes-monocytes and fewer granulocytes during the first week after mixing when compared with pigs in the other 2 treatments ($P < 0.02$). No treatment differences were observed in the ability of macrophages to respond to an LPS challenge with the production of nitric oxide ($P = 0.82$, data not shown). When macrophages were challenged with 10 μg of LPS, nitric oxide production was 332.8 ± 5.6, 329.3 ± 4.8, and 326.8 ± 5.1 μ*M* for pigs in the control, ACTH, and rough treatments, respectively. Similar responses were found when macrophages were challenged with 20 μg of LPS. The antibody response to oRBC did not differ among treatments ($P = 0.65$). All pigs had a mean titer (agglutination, $\log_2$) of 2.03 ± 0.34 on d 0; had their peak response to the first injection on d 11, with 4.47 ± 0.31; and peaked in response to their second vaccination, with 7.65 ± 0.55 on d 42 (the last day this was measured).

DISCUSSION

We found it interesting that prenatal stress, in the form of rough handling, actually improved the ability of the offspring to cope with the LPS challenge. One theory of the teleological function of prenatal stress is that it serves to prepare offspring for their future environment, based on the stress their dam experienced during their gestation. Many studies in the rodent literature (e.g., Ward, 1984; Henry et al., 1994; Vallée et al., 1996) that invoke prenatal stress have found that offspring have a heightened HPA axis response to stressors. A heightened HPA response can be adaptive in that it helps the offspring respond appropriately to stressors in the environment (Levine and Mullins, 1968). Indeed, Lambert et al. (1995) found that prenatally stressed rats developed less ulceration when they were subjected to stress at 50 d of age. Furthermore, research in European wild rabbits (Cabezas et al., 2007) has shown that rabbits responding to a chronic stressor with a greater gluco-

Table 1. Mean hematological data for pigs in response to mixing[1]

Blood variable[2]	Unit	Treatment			*P*-value	
		ACTH	Control	Rough	Treatment	Treatment × time
Hematocrit	%	37.70 ± 0.25	37.00 ± 0.29	37.23 ± 0.29	0.4151	0.7813
Hemoglobin (Hgb)	g/dL	12.70 ± 0.08	12.49 ± 0.09	12.58 ± 0.09	0.4668	0.7386
Mean corpuscular Hgb	g/dL	33.72 ± 0.07	33.83 ± 0.07	33.81 ± 0.07	0.8218	0.1177
White blood cells	billions/L	18.55 ± 0.36	18.36 ± 0.34	17.89 ± 0.36	0.7441	0.3733
Granulocytes	billions/L	9.70 ± 0.26	9.89 ± 0.24	9.98 ± 0.26	0.6566	0.3041
Granulocytes	%	51.64 ± 0.82	53.87 ± 0.76	55.55 ± 0.79	0.0196	0.0119
Neutrophils	billions/L	8.08 ± 1.61	9.14 ± 0.58	7.75 ± 0.51	0.8229	0.5719
Eosinophils	billions/L	2.11 ± 0.33	1.96 ± 0.14	2.00 ± 0.13	0.7977	0.2366
Lymphocytes-monocytes	billions/L	9.12 ± 0.4	8.47 ± 0.21	7.91 ± 0.21	0.0756	0.0883
Lymphocytes-monocytes	%	47.80 ± 0.73	46.13 ± 0.76	44.45 ± 0.79	0.0349	0.0193
Platelet count	billions/L	340.25 ± 10.24	320.30 ± 10.47	349.87 ± 8.96	0.2038	0.3202
Reticulocytes	%	1.07 ± 0.05	1.16 ± 0.06	1.22 ± 0.06	0.2195	0.1380

[1]n = 14 for the ACTH treatment, n = 15 for the control treatment, and n = 14 for the rough treatment.

[2]Data are presented as the overall means for the 10 blood samples collected during the 35 d after test pigs were placed into a pen of 5 other unfamiliar individuals.

corticoid response actually had a greater survival rate in the wild. However, in our study, pigs in the rough treatment did not have greater salivary cortisol; thus, a heightened HPA response does not appear to be the method by which prenatal stress can enhance the ability of the pigs to cope with the LPS challenge.

Our findings are in agreement to those of de Groot et al. (2007), who also found that pigs from sows having received HCA during gestation expressed sickness behavior for a shorter duration in response to the LPS challenge used in this study. However, this finding was true only for pigs from dams who received treatments during midgestation (d 51 to 80 of gestation) as compared with dams receiving treatments in either early (d 21 to 50) or late (d 81 to 110) gestation. Pigs from dams treated in midgestation also mounted a less pronounced febrile response to the LPS challenge. The febrile response and sickness behavior are intimately associated because the LPS challenge will activate macrophages to release IL-1, causing stimulation of corticotrophin releasing factor and ACTH, and the onset of sickness behavior (see Hart, 1988, for a review). In agreement with our study as well, these authors did not find differences in the salivary cortisol response of pigs to the LPS challenge. In light of these findings, it is unclear why salivary cortisol was not increased, implying that the changes in behavior are being regulated in the central nervous system through measures that we cannot observe in the peripheral system.

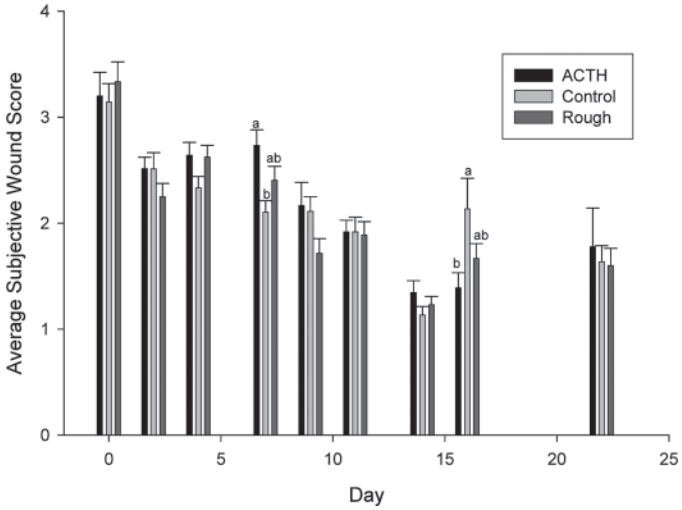

Figure 3. Mean (±SE) biopsy puncture scores evaluated from photographs and averaged from 3 observers blinded to the treatments. Punctures were evaluated on a scale of 1 to 4, with 1 indicating no inflammation and 4 indicating a deep red ring around the wound with pus. n = 14 for the ACTH treatment, n = 15 for the control treatment, and n = 14 for the rough treatment. [a,b]Means with different letters differ ($P < 0.05$).

Another curious consideration is that the pigs in our rough treatment, but not those in the ACTH treatment, were better able to cope with the LPS challenge. Our ACTH treatment would be more analogous to the treatment used by de Groot et al. (2007) of oral administration of HCA in that both increase plasma cortisol. This is in contrast to our rough treatment, which brings with it a host of possible physiological responses associated with a real stressor that encompasses psychological stress. Of course, what both treatments have in common is that oral administration of HCA and rough handling will increase plasma cortisol, and it could be that the greater increase in plasma cortisol in sows in the ACTH treatment (previously reported by Lay et al., 2008) did not produce the same prenatal changes as when cortisol is increased to a lesser degree.

In this study, we found that prenatal stress did not alter either pCBG or plasma cortisol in swine. This in contrast to several studies (including one by the authors of the current study) that have found either increases in cortisol in response to stress (Haussmann et al., 2000; Jarvis et al., 2006) or decreases in cortisol in response to exogenous ACTH (Kranendonk et al., 2006b). However, data from the current study are in agreement with several other studies that did not find changes in cor-

tisol or pCBG in response to stressors (Otten et al., 2001, to exogenous ACTH; de Groot et al., 2007, to LPS challenge; Lay et al., 2008, to weaning; Couret et al., 2009, to a novel environment or castration; Otten et al., 2010, to weaning or relocation).

In our previous prenatal stress research (Haussmann et al., 2000), we found behavioral differences in pigs, with control pigs expressing greater oral (vice) behaviors and a tendency to belly nose more. Thus, in the present experiment, we explored whether behavioral differences might be seen in older pigs. However, no differences were found in activity or aggressive behavior during mixing. This could be because the relatively stressful event of mixing masked any subtle differences that may have been present.

Most studies on prenatal stress in swine have focused on the HPA axis of the neonatal pig, with limited emphasis on older pigs or their immune capability. Thus, the focus of this research was to examine the interaction between the HPA response of pigs and immune system function. Instead of simply measuring immune cell populations or using an in vitro test of immune cell activity, we wanted to subject pigs to real immune challenges that occur in production agriculture. Punch biopsy data indicating that pigs in the ACTH treatment were slower to heal the wound on d 7 is in agreement with our earlier research (Haussmann et al., 2000). However, the reverse being true on d 16 in the current study suggests that the impairment of pigs in the ACTH treatment was due to a heightened sensitivity to stress around d 7. Whether this reversal would have happened in the study by Haussmann et al. (2000) is unclear because the measurements were taken only through d 11. The reverse effect on d 16 was not due to the pigs in the ACTH treatment advancing their healing process, but instead to the control pigs actually regressing back to the wound score they had on d 7. This could be a natural regression in the wound-healing process because the wound scores of pigs in all treatments were numerically increased.

Pigs in our study did not exhibit many alterations in their hematology measures, ability to mount an antibody response to a foreign pathogen, or ability of their macrophages to produce nitric oxide. This was an unexpected result, but in view of the limited effect of the prenatal stress treatments on HPA axis function, it is not surprising. Although it would appear that the immune function itself is not driven by changes in HPA axis activity, it is clear that HPA axis activity has a broad impact on many variables of the immune system.

Our results suggest that although prenatal stress clearly has interesting effects on subsequent offspring, no simple cause-and-effect relationship exists. Our data, and those of others, indicate that this is not a robust phenomenon in swine that is capable of transcending differences in environment, genetics, or the quality or severity of stressors that are used in swine research. The literature on rodents, although also at times inconsistent, is much more consistent than the literature we have for swine. In part, this could be due to the relatively limited number of swine studies. However, the authors surmise that large differences in placental type between rodents and swine are the reason for the incongruent results between the 2 species. Swine have an epitheliochorial placenta composed of 6 tissue layers, whereas rodents have a hemochorial placenta composed of only 3 tissue layers of separation. The more significant barrier of the swine placenta may be preventing the passage of compounds that cause prenatal stress. It is encouraging that our results replicate those of de Groot et al. (2007) relative to the very intriguing finding of prenatal stress on what appears to be enhancement of the ability of a pig to cope with an infection behaviorally.

The results from the body of research that investigates prenatal stress in swine illuminates several areas of future research that need to be pursued. The lack of consistent results among laboratories, as well as within laboratories, is informative. Instead of indicating that prenatal stress is incomprehensible, we believe it is showing how individual uniqueness modifies or eliminates the effects of prenatal stress. With the many environmental factors identified above, individual phenotypes are further defined by the type and their perception of a stressor. Factor analyses using the multitude of factors identified may be fruitful in elucidating phenotypes that are resistant to or compliant with the effects of prenatal stress. The development of a livestock model for prenatal stress would also serve to advance our understanding of the mechanisms involved. For instance, Lay and Wilson (2002) proposed using the chicken to study the effects of prenatal stress. The chicken provides a unique opportunity for researchers to manipulate the developing fetus without confounding the effects derived from the maternal exchange of compounds that occurs in mammals. A final area of research that shows great potential focuses on the communication between the stress response (HPA axis and sympathoadrenal axis) and the immune system. It is well established that activation of the stress response causes a multitude of responses in the immune system (for a review, see Webster et al., 2002; Padgett and Glaser, 2003). Significant components of this response are changes in cytokine activity, which can influence HPA activity and prenatal development. For instance, it is known that IL-6 is able to cross from the maternal circulation to the fetus in midgestation (Dahlgren et al., 2006), and that IL-6 derived from maternal circulation causes deficits in the behavioral response of adult mice to cope with stress (Smith et al., 2007) and decreases learning in adult rats (Samuelsson et al., 2006). Interestingly, human patients diagnosed with depression have been shown to have serum IL-6 concentrations that are 10 times greater than those of control patients (Berk et al., 1997). It would be interesting to determine if the affective state of livestock or chronic exposures to stress could be regulating responses through cytokine modulation. Because glucocorticoids have overt effects

on cytokine regulation, future research to understand how cytokines regulate normal brain development will shed light on how prenatal stress causes differential effects on developing livestock.

LITERATURE CITED

Adcock, R. J., H. G. Kattesh, M. P. Roberts, J. A. Carroll, and A. M. Saxton. 2006. Relationships between plasma cortisol, corticosteroid-binding globulin (CBG) and the free cortisol index (FCI) in pigs over a 24 h period. J. Anim. Vet. Adv. 5:85–91.

Berk, M., A. A. Wadee, R. H. Kuschke, and A. O'Neill-Kerr. 1997. Acute phase proteins in major depression. J. Psychosom. Res. 43:529–534.

Cabezas, S., J. Blas, T. A. Marchant, and S. Moreno. 2007. Physiological stress levels predict survival probabilities in wild rabbits. Horm. Behav. 51:313–320.

Chou, S. H., L. Kojic, K. Nordyke Messngham, and J. E. Cunnick. 1996. Characterization of the effect of 2-deoxy-D-glucose on the immune system. Brain Behav. Immun. 10:399–416.

Couret, D., A. Prunier, A.-M. Mounier, and F. Thomas. 2009. Comparative effects of a prenatal stress occurring during early or late gestation on pig immune response. Physiol. Behav. 98:498–504.

Dahlgren, J., A.-M. Samuelsson, T. Jansson, and A. Holmäng. 2006. Interleukin-6 in the maternal circulation reaches the rat fetus in mid-gestation. Pediatr. Res. 60:147–151.

Daniel, J. A., D. H. Keisler, J. A. Sterle, R. L. Matteri, and J. A. Carroll. 1999. Birth by caesarian section alters postnatal function of the hypothalamic-pituitary-adrenal axis in young pigs. J. Anim. Sci. 77:742–749.

de Groot, J., G. Kranendonk, M. Fillerup, H. Hopster, W. Boersma, D. Hodgson, K. van Reenen, and M. Taverne. 2007. Response to LPS in female offspring from sows treated with cortisol during pregnancy. Physiol. Behav. 90:612–618.

Hart, B. L. 1988. Biological basis of the behavior of sick animals. Neurosci. Biobehav. Rev. 12:123–137.

Haussmann, M. F., J. A. Carroll, G. D. Weesner, M. J. Daniels, and D. C. Lay Jr. 2000. Administration of ACTH to restrained pregnant sows alters their pigs' hypothalamic-pituitary-adrenal axis. J. Anim. Sci. 78:2399–2411.

Henry, C., M. Kabbaj, H. Simon, M. Le Moal, and S. Maccari. 1994. Prenatal stress increases the hypothalamic-pituitary-adrenal axis response in young and adult rats. J. Endocrinol. 6:341–345.

Jarvis, S., C. Moinard, S. K. Robson, E. Baxter, E. Ormandy, A. J. Douglas, J. R. Seckl, J. A. Russell, and A. B. Lawrence. 2006. Programming the offspring of the pig by prenatal social stress: Neuroendocrine activity and behaviour. Horm. Behav. 49:68–80.

Kanitz, E., W. Otten, M. Tuchscherer, and G. Manteuffel. 2003. Effects of prenatal stress on corticosteroid receptors and monoamine concentrations in limbic areas of suckling piglets (*Sus scrofa*) at different ages. J. Vet. Med. A Physiol. Pathol. Clin. Med. 50:132–139.

Kranendonk, G., H. Hopster, M. Fillerup, E. D. Mulder, and M. A. M. Taverne. 2006a. Cortisol administration to pregnant sows affects novelty-induced locomotion, aggressive behaviour, and blunts gender differences in their offspring. Horm. Behav. 49:663–672.

Kranendonk, G., H. Hopster, M. Fillerup, E. D. Mulder, V. M. Wiegant, and M. A. M. Taverne. 2006b. Lower birth weight and attenuated adrenocortical response to ACTH in offspring from sows that orally received cortisol during gestation. Domest. Anim. Endocrinol. 30:218–238.

Lambert, K. G., C. H. Kinsley, H. E. Jones, S. L. Klein, S. N. Peretti, and K. M. Stewart. 1995. Prenatal stress attenuates ulceration in the activity stress paradigm. Physiol. Behav. 57:989–994.

Lay, D. C., Jr., H. G. Kattesh, J. E. Cunnick, M. J. Daniels, K. A. McMunn, M. J. Toscano, and M. P. Roberts. 2008. Prenatal stress effects on pig development and response to weaning. J. Anim. Sci. 86:1316–1324.

Lay, D. C., Jr., and M. E. Wilson. 2002. Development of the chicken as a model for prenatal stress. J. Anim. Sci. 80:1954–1961.

le Roux, C. W., S. Sivakumaran, J. Alaghband-Zadeh, W. Dhillo, W. M. Kong, and M. J. Wheeler. 2002. Free cortisol index as a surrogate marker for serum free cortisol. Ann. Clin. Biochem. 39:406–408.

Levine, S., and R. F. Mullins Jr. 1968. Early experience and behavior: The psychobiology of development. Pages 168–197 in Hormones in Infancy. G. Newton and S. Levine, ed. Charles C. Thomas, Springfield, IL.

Otten, W., E. Kanitz, D. Couret, I. Veissier, A. Prunier, and E. Merlot. 2010. Maternal social stress during late pregnancy affects hypothalamic-pituitary-adrenal function and brain neurotransmitter systems in pig offspring. Domest. Anim. Endocrinol. 38:146–156.

Otten, W., E. Kanitz, M. Tuchscherer, and G. Nürnberg. 2001. Effects of prenatal restraint stress on hypothalamic-pituitary-adrenocortical and sympatho-adrenomedullary axis in neonatal pigs. Anim. Sci. 73:279–287.

Padgett, D. A., and R. Glaser. 2003. How stress influences the immune response. Trends Immunol. 24:444–448.

Roberts, M. P., H. G. Kattesh, G. A. Baumbach, B. E. Gillespie, J. D. Godkin, J. F. Schneider, and A. M. Saxton. 2003. Age-related changes in porcine corticosteroid-binding globulin (pCBG) as determined by an enzyme-linked immunosorbent assay. Domest. Anim. Endocrinol. 24:323–339.

Samuelsson, A.-M., E. Jennische, H.-A. Hansson, and A. Holmäng. 2006. Prenatal exposure to interleukin-6 results in inflammatory neurodegeneration in hippocampus with NMDA/$GABA_A$ dysregulation and impaired spatial learning. Am. J. Physiol. Regul. Integr. Comp. Physiol. 290:R1345–R1356.

Smith, S. E. P., J. Li, K. Garbett, K. Mirnics, and P. H. Patterson. 2007. Maternal immune activation alters fetal brain development through interleukin-6. J. Neurosci. 27:10695–10702.

Snyder, M. L., K. A. Eernisse, D. R. Jutting, and L. A. Middle. 1981. Microtitration hemagglutination inhibition test for swine influenza virus (SIV). Pages 32–34 in Serologic Microtitration Techniques. USDA, Animal and Plant Health Inspection Service, Veterinary Services, National Veterinary Services Laboratories, Ames, IA.

Vallée, M., W. Mayo, S. Maccari, M. Le Moal, and H. Simon. 1996. Long term effects of prenatal stress and handling on metabolic parameters: Relationship to corticosterone secretion response. Brain Res. 712:287–292.

Ward, I. L. 1984. The prenatal stress syndrome: Current status. Psychoneuroendocrinology 9:3–11.

Webster, J. I., L. Tonelli, and E. M. Sternberg. 2002. Neuroendocrine regulation of immunity. Annu. Rev. Immunol. 20:125–163.